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RECENT HPLC STRATEGIES TO IMPROVE SENSITIVITY AND SELECTIVITY FOR THE ANALYSIS OF COMPLEX MATRICES

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RECENT HPLC STRATEGIES TO IMPROVE SENSITIVITY AND SELECTIVITY FOR THE ANALYSIS OF COMPLEX MATRICES

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□ *Recently, various approaches have been addressed to improve chromatographic performance in terms of sensitivity or selectivity, from the development of novel on-line (or off-line) enrichment methodologies, to the improvement of stationary-phase type and multidimensional chromatography, to novel and more performing detectors such as tandem mass spectrometry (MS/MS), nuclear magnetic resonance (NMR), and infrared spectrometry (IR). For the analysis of complex matrices (e.g., biological fluids, plant extracts, food, and environmental samples), coupling chromatographic instrumentation with these detectors provides a powerful analytical device that can be applied in many fields, such as analysis of pharmaceutical and natural products (and medicinal plant), quality control, and environmental trace analysis.*

This review describes major advances in the HPLC field in terms of enrichment techniques and chromatographic separation, and especially in terms of detector improvement for a complete identification and quantification of targeted analytes. The possibility of high-throughputs analyses, as a consequence of sensitivity and selectivity enhancement in drug and metabolites profile and multi-residue assays for natural compounds and/or products containing natural species, is also highlighted. Finally, it is shown how multivariate analysis may enhance analytical performance in terms of useful analytical information that can be extracted from experimental data and in terms of methods for exploring and modeling data. Chemometrics provides tools for making the most of analytical signals, once selectivity and sensitivity have been improved from the chemical point of view.

Keywords chemometrics, discriminant analysis, drug and metabolites, enrichment and purification techniques, HPLC configuration, HPLC detectors, multivariate classification and calibration, natural product analysis, PCA, PCR, PLS, SIMCA

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INTRODUCTION

High performance liquid chromatography (HPLC) offers several advantages in terms of chromatographic resolution, fast analysis, small sample volume, and low organic solvent consumption (when the chromatographic separation is achieved following green-chemistry principles[1]). It is a powerful assay suitable for the analysis of biological samples, especially when a small amount of sample is available, as in the case of pharmaceutical and biomedical fields. For these reasons, this technique, like capillary electrophoresis as recently reviewed by Locatelli and Carlucci,[2] is suitable to quantify drugs, metabolites, and biomarkers in biological fluids where limit of detection (LOD) and/or limit of quantification (LOQ) at lower mg/L range is required.

A basic issue in method development is optimizing sensitivity and selectivity. Bioanalytical method validation guidelines[3–7] require these quality parameters to be evaluated when a new HPLC method is validated.

For several years, reversed-phase high-pressure liquid chromatography (RP-HPLC) has dominated the applications and methods that were developed, but now it is necessary to improve stationary phases or detector performance in terms of selectivity and sensitivity in order to face very difficult separations and determinations when real samples and complex matrices are involved.

Routine detectors for HPLC separation are ultraviolet-visible (UV/Vis), fluorescence (FLD), refractive index (RID), and electrochemical (ECD) detectors. In the last decade, Fourier transform-infrared (FT-IR) and mass spectrometry were extensively used as detection techniques. The detection limits and merits of HPLC detectors are listed in Table 1.

Coupling fluorescence detection with HPLC generally improves sensitivity and selectivity with respect to HPLC-UV configuration, since the intensity of the emitted fluorescent light is related to the intensity of the incident light. Then, the detection limit can be improved by opportunely setting instrumental parameters relevant to excitation light. In this way, three different approaches to fluorescence detection are possible, involving measurements of indirect fluorescence or native fluorescence, and fluorescence of derivatized analytes.

The coupling of HPLC with ECD, which are extremely selective, is especially suited to separation, being based on a reaction at an electrode surface. LODs often decrease when the electrode size is reduced. ECD detectors provide four possible signal-acquisition modes: amperometric, pulsed amperometric, potentiometric, and conductometric. In potentiometric and conductometric configuration, the potential (or conductivity) between electrodes is measured as the analytical signal, while in pulsed amperometric detection (PAD) a three-step waveform is

TABLE 1 Detection Limits and Merits of HPLC Detectors

Detection Method		Mass Detection Limits*	Note For Analytical Application
UV absorption		1 pg–1 ng	Relatively sensitive Not selective Direct and indirect
Fluorescence		10 fg–10 pg	Sensitive Selective Native detection or pre-column derivatization
RID		10 ng–1 µg	Relatively sensitive Not selective
Electro-chemical	Amperometric	100 fg–1 ng	Sensitive Selective (can be turned to the analyte of interest)
	Conductometric	500 pg–1 ng	Moderate sensitive Universal detection
	Potentiometric	Not reported	Not reported
FT-IR		100 ng–1 µg	Relatively sensitive Structural elucidation
Mass spectrometry (in HPLC-ESI-MS configuration)		100 ag–1 ng	Sensitive Selective (in MS/MS mode) Structural determination (in high resolution mode, e.g., FT-ICR-MS) Metabolic, proteomic, and ADME studies

*LOD values are expressed in injected mass that brings to a signal-to-noise ratio of 5-folds, using 200 g/mol as molar mass with an injection volume of 10 µL.

applied (corresponding to detection, oxidative cleaning, and reactivation, respectively).

Selectivity (high separation efficiency) and sensitivity (as reported in Table 1) of HPLC-ECD allow application of this configuration to analyses of drugs and related metabolites in biological fluids, especially considering the low sample amount required (5–20 µL).

High performance liquid chromatography–mass spectrometry (HPLC-MS) is an analytical technique that couples high-resolution chromatographic separation with sensitive and specific mass-spectrometric detection. A new ionization source, electrospray (ESI), introduced and validated by Fenn et al.[8,9] to interface HPLC instrumentation with a mass spectrometric detector, gave a great impulse to this hyphenation. In this mode, the sample is introduced in a flowing stream. Since the flow discharges from the tiny nozzle held at high voltage with respect to the ion source lens, droplets emerge bearing the electrolyte on the surface and carrying the sample within. While the solvent is stripped away, the droplet size decreases and sample-rich particles result, often bearing high numbers of charges. When the charged molecular ions enter into the analyzer, they bear little energy excess and are quite stable. ESI

mass-spectra mostly contain molecular ions in a distribution of charge states. ESI is a "soft" ionization technique in that poor fragmentation is obtained, forming protonated or de-protonated ions for positive and negative ionization mode, respectively. The last acquisition mode is less sensitive, except when the native analyte forms stable anions, such as carboxylic acids. The development of ESI led to a great increase in the use of HPLC-MS, proving to be an ionization technique that is highly compatible with solvents generally used in RP-HPLC, and particularly efficient for polar compounds.

A very interesting review by Arslan et al.[10] reports the advantages derived from coupling HPLC with the chemical-vapor generation detector technique especially related to the LOD improvement in speciation analyses.

FT-IR is another detector used in HPLC for the analyses of complex matrices. Based on the absorption of IR radiation, HPLC-IR (or FT-IR) permits the improvements of LOD and LOQ by acquisition of analyte-specific chromatograms (with lower noise level) and the identification of unknown materials by its specific interferogram.

During the past 20 years, the techniques discussed here have been intensively developed, particularly in the mass-spectrometry field, and a strong improvement in sensitivity and resolution has been achieved.

ENRICHMENT AND PRETREATMENT TECHNIQUES (ON-LINE AND OFF-LINE)

Several methodologies have been developed for increasing HPLC sensitivity and selectivity without the need for more expensive or complex detection systems, such as MS. The principal methodologies are based on a preconcentration step prior to analysis; this is the case for solid-phase extraction (SPE) (both off-line and on-line), solid phase micro extraction (SPME), micro extraction by packed sorbent (MEPS), and other techniques such as derivatization (both off-line and on-line) and membrane-based extraction methodologies. These procedures permit one to increase the HPLC potentiality in several fields, especially when low analyte and metabolite concentrations are present.

The reduction of analyte losses and the possibility to analyze even a total sample (no loss) leads to lower limits of detection (and consequently lower limits of quantification). Moreover, smaller sample volumes may be used to obtain adequate sensitivity and selectivity for a large variety of compounds. In addition, on-line SPE requires low solvent consumption. On-line SPE coupled to HPLC also presents a strong advantage: it is not necessary to remove all residual water from cartridges, since elution solvents are compatible with the HPLC separation methods.

Arthur and Pawliszyn in the 1990s[11] introduced SPME as a fast, solvent-free extraction technique, which has been widely applied to many applications: biological, environmental, etc. For the SPME approach, selection of a suitable fiber is crucial: even if there are several commercial fibers, most of them generally suffer from some drawbacks, such as high cost, chemical instability, and poor robustness. All these issues have long limited the application of SPME to real matrices.[12–14] To overcome these drawbacks, Wu and Pawliszyn[15] applied the electrochemical coating approach. SPME is an adsorption technique,[11] and the extraction capacity is low because few adsorption sites on the fibers[16] are generally present. In recent years, there has been an increasing interest in the application of nano-structured SPME fibers, due to their unique properties, such as large specific surface area, multiple active sites for adsorbing analytes, and extraction capacity of nano-structured fibers.[17]

To overcome drawbacks encountered in SPME, in recent years novel micro extraction instrumentation was developed and validated for several pharmaceutical applications. MEPS can be used both on-line and off-line, and, in particular, they can be applied for HPLC and GC analyses. This technique offers several advantages deriving from SPME and SPE extraction and purification protocols. The MEPS approach to sample preparation is suitable for reversed phases, normal phases, mixed mode, or ion-exchange chemistries. MEPS is available in a large variety of common SPE phases. It reduces the time needed to prepare and inject samples from hours to minutes: the elimination of all extra steps between sample preparation and sample injection reduces any analyte loss, reduces the buffer and solvent volume from mL to µL (according to green chemistry concepts[1]), while the needed sample volume may be lower than 3.6 µL (Figure 1).

The MEPS extraction instrumentation presents strong improvements in terms of recovery and packed sorbent stability; it was published by Abdel-Rehim in an interesting review on this novel micro-extraction technique based on packed sorbent for bioanalysis.[18]

Recently, increased performance in sample preparation was obtained by SPE on-line coupled with HPLC instrumentation, as reported in Figure 2.

Higher sensitivity is also achieved in on-line configurations: this is due to the transfer and analysis of the whole extracted species. On the contrary, in off-line SPE procedures, only an aliquot of the extract is injected into the column.[19]

Derivatization is the most used technique when the analytes do not show any chromophores and electrophores and the final goal is to detect "invisible" molecules, and it is often used also with polar or ionic species due to the extraction difficulties. In addition, the derivatization step permits the stabilization of labile molecules.[20] This technique is often used especially for the improvement of sensitivity (5–2000-fold) and selectivity due to the tagged

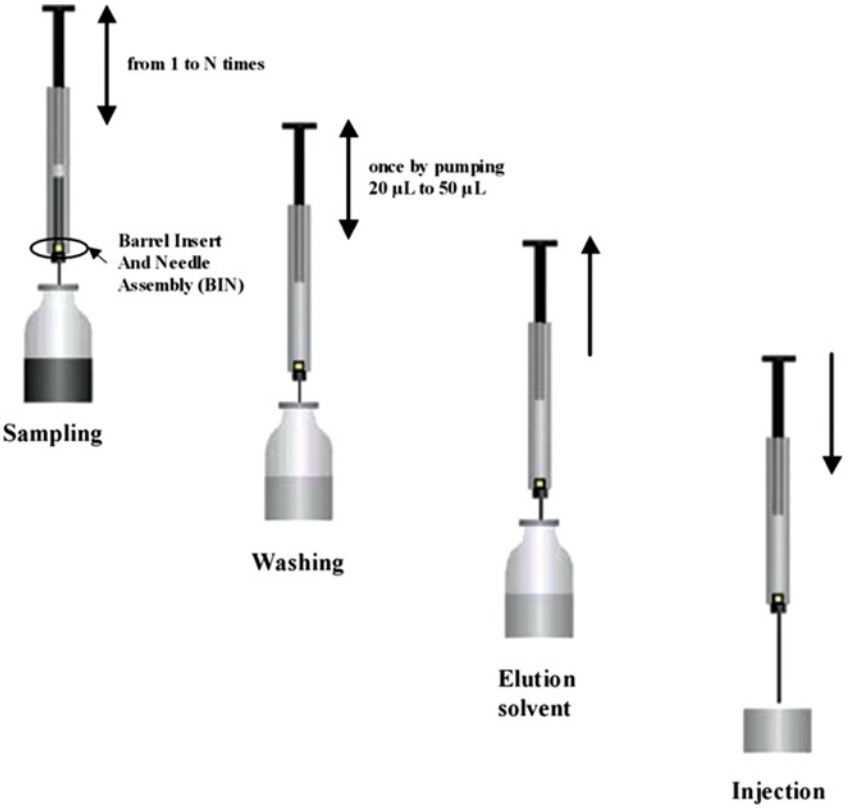

FIGURE 1 MEPS extraction procedure. (color figure available online.)

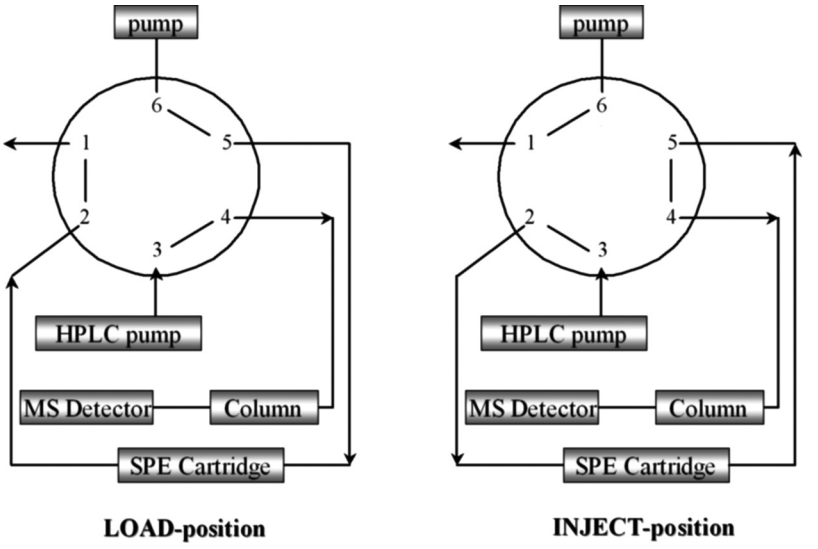

FIGURE 2 System diagram used for on-line SPE–HPLC–MS.

functional groups. The main advantage of this procedure is that it can be done both off-line and on-line, like SPE.[19] Several derivatization processes were developed and reported and discussed by Rosenfeld,[21a] in particular the DANSYLation or specific derivatization used in HPLC-ESI-MS.

MEMBRANE-BASED EXTRACTION TECHNIQUES

Membrane-based processes are very attractive, especially for applications that need a continuous monitoring in environmental and clinical fields. They are based on permeation phenomena through a membrane, and specific sorption (into) and desorption (out of) processes occur simultaneously. In this process the mass transport rate is generally controlled by analyte diffusion. Several techniques based on this principle were developed such as dialysis (and microdialysis), membrane extraction with solvent interface (MESI), and gas-diffusion approach.

Dialysis and microdialysis were accomplished using a probe with a semipermeable membrane. A solution with a similar ionic strength of the external solution (sample) flows through the probe, producing a concentration gradient between the perfusate and the surrounding medium. In this way, the solution from the probe (dialysate), enriched in analyte molecule, is delivered to the instrument.

These techniques are especially useful in clinical field for drug metabolism and distribution, pharmacokinetic processes, and environmental monitoring, as reported by Nandi and Lunte.[21b] The main problem related to these methodologies is the calibration.

To obtain a process control, the MESI method was developed. In MESI, an internal and non-interfering calibrant is added in dialysate. The changes in relative internal calibrant loss can be used to control and calibrate the system.[21c]

Another interesting application of semi-permeable membrane was represented by gas-diffusion extraction (and micro-extraction) techniques. With a micro-porous gas-permeable membrane in a probe, the analysis in HPLC is possible using the previously cited detector. This is a novel and recent extraction procedure developed especially for the analysis of volatile and semi-volatile compounds in various matrices, such as beer,[22] and if coupled on-line with derivatization processes, a considerable enrichment of factors can be obtained.

STATIONARY PHASE IMPROVEMENTS

The most common choice for improving chromatographic performance is the use of columns packed with low-diameter particles

(sub-2 μm particles)[23] to obtain efficiency improvement, optimal velocity, and mass transfer. The main issue in using low-diameter stationary-phase packing is that pressure increases with the square of the particle diameter. It is common to prepare conventional columns packed with particles in the 1–2 μm range, and to operate at high linear velocities. These two conditions cause a very high inlet pressure. Most commercial HPLC instruments have 400 bars as maximum limit of operating pressure—this involves the use of short columns packed with 1–2 μm particles to speed up analysis.[24] Several manufacturers since 2004 have introduced a new generation of columns packed with sub-2 μm porous particles[25] that offer reliable performance with respect to conventional particle sizes.[26] In the meantime, analytical devices operating at pressures higher than 400 bars (such as the UPLC) have been commercialixed.[27] The term ultra-high pressure liquid chromatography (UHPLC) is especially related to the higher backpressure requirement (>400 bars).[28] The combination of uniform particle shape, narrow particle-size distribution, and significantly shorter diffusion path results in higher column efficiencies and, consequently, increased chromatographic resolution. The increased efficiencies also induce immediate benefits in terms of sensitivity: higher chromatographic efficiencies bring significantly narrower and taller peaks, allowing detection at lower analyte-concentration levels. More than efficiency, selectivity is the most important parameter to increase resolution.

Innovations in HPLC particle technology are addressed to improved chromatographic performance and higher productivity. To achieve performance improvements such as greater sensitivity, higher resolution (and consequently higher selectivity), and faster analysis times, a column requires lower plate height at a wide range of linear velocities. In the case of traditional fully porous 3–5 μm particles, efficiency decreases significantly as flow rate increases. In most cases, loss of resolution (and selectivity) and sensitivity prevents faster analysis times. Smaller fully porous particles (< 2 μm) generally provide faster chromatographic separations at lower plate heights (height equivalent to a theoretical plate (HETP)), but require instrumentation operating at higher pressure.

Available sub-2 μm columns generally provide roughly threefold the efficiency with respect to 5-μm fully porous particles and twofold the efficiency compared to 3-μm fully porous particles.

Monolithic columns provide an alternative approach to fast HPLC separations without compromising efficiency or resolution (selectivity). The high permeability and large number of theoretical plates per pressure-drop unit, associated with monolithic stationary phase, derive from fundamental physical features: large $\left(\frac{\text{through - pore size}}{\text{skeleton - size}}\right)$ ratio and high porosities. The higher porosity and smaller skeleton size of monolithic

columns permits operation at higher flow-rates on relatively long columns with conventional HPLC instruments, thus achieving high-speed and high-efficiency separations.

The most used stationary phases in capillary UHPLC are generally 1–1.5 μm silica-based nonporous particles.[29] A major advantage was achieved by reduction of band broadening due to sample diffusion related to mass transfer within the pores. It was observed that nonporous particles generally provide overall higher efficiency at high linear velocities; the efficiency gain diminished when the particle size was reduced from 3 to 1.5 μm. A very important point related to sensitivity is that, for porous C_{18} particles, the capacity, that is maximum sample loading, was 15 times higher compared to non-porous C_{18} particles. Moreover, for 1.7-μm porous C_{18} column, the average retention factor is significantly higher with respect to 1.5 μm nonporous C_{18} column. These effects are due to the fact that mobile phases with higher aqueous content are required to obtain similar retention factors, so pressure drop for nonporous particles is higher than for porous ones.[30]

A very interesting review by Tao et al.[31] reports the latest advances in micro-scale and nano-scale HPLC, also in configuration with very sensitive and selective detectors.

Another improvement in stationary phases was related to the development, by Kirkland, of "fused-core" technology. This type of stationary phase has several advantages, especially due to the fact that the diffusional mass transfer path is reduced. It allows the use of shorter columns, and consequently, it is possible to use higher flow rates. In this way, with particle sizes lower than 3 μm (generally 2.7 μm divided into 1.7 μm of solid core and 0.5 μm of external porous layer), short column, and high flow rate, the overall chromatographic performances (efficiency) are increased.[32]

MULTIDIMENSIONAL HPLC

First theoretical descriptions and experimental accounts of multidimensional chromatographic methods were set up in the 1970s and 1980s,[33] especially related to the higher resolving power of these methods. Almost all of these improvements were developed within the bounds of two-dimensional (2D) separations. Multidimensional liquid chromatographic (MDLC) methods can be divided into two main groups: comprehensive separations (denoted LC×LC for a two dimensional separation), relevant to separation and quantification of a large number (also up to thousands) of sample constituents,[34] and "heartcutting" or "coupled column" methods (LC–LC for a two-dimensional separation)

are instead considered "targeted methods," generally focused on the separation, identification, and quantification of several sample-matrix constituents.

Research in the LC×LC field has increased in the past three years, and currently separations of several hundred constituents in one sample on the one-hour total run are not rare.[35] MDLC has been successfully applied to the analysis of a large variety of trace-level analytes in complex mixtures. Several reviews regarding the versatility of "targeted MDLC" approaches, and different instrument configurations and applications areas have been published.[36–38] Recent improvements of instrument configuration have been achieved in noteworthy selectivity, sensitivity, and quantification level with and without the use of more expensive detection modes.[39] To our knowledge, the most elaborate and powerful three-dimensional (3D) separation to date was addressed to a comprehensive 3D separation group, and was used to demonstrate the separation of hen ovalbumin peptide fragments using size exclusion (first dimension), reversed-phase chromatography (second dimension), and capillary zone electrophoresis (third dimension).[40]

The use of 3D-MDLC systems would also have benefits over 1D and 2D systems where ESI-MS detection is required. The increased separation power helps to reduce ion suppression effects due to co-eluting compounds in electrospray ionization[41] and consequently to reduce the matrix effects in ESI source. Simpkins et al.[42] recently published a work concerning the utility of "targeted 3D-HPLC" for the simplification of analysis of a wide range of compounds.

ROLE OF DETECTION IN IMPROVING SENSITIVITY AND SELECTIVITY

Tandem Mass Spectrometry (MS/MS) and Nano-ESI

The selectivity and sensitivity (as reported in Table 1 Table 1 first mention) of HPLC-MS (and in particular HPLC-MS/MS) permit the application of this hyphenation to the analysis of drugs, metabolites, and biomarkers in biological fluids for pharmacokinetic, adsorption distribution metabolism and elimination (ADME), and bioequivalence studies. The ESI-MS/MS approach is usually preferable, because it allows precursor ion selection and better control in the decomposition process. Although single and triple quadrupole instruments dominate the ESI literature, ESI may also be performed with ion traps, time-of-flight (ToF), or multi-sector mass analysers, such as Q-ToF and Q-Trap. With ion traps, both quadrupolar and Fourier transform–ion cyclotron resonance

(FT-ICR), multiple stages of mass selection and decomposition are possible, as well as studies of ion-molecule reactions.

In this field, a very important instrumentation is triple quadrupole (QqQ), since it permits several kinds of analysis, such as multiple reactions monitoring (MRM) mode, neutral loss, neutral gain, parent ion scan and daughter ion scan. The most applied data acquisition mode is certainly MRM (multiple reactions monitoring) type, where molecules may be detected with higher specificity and sensitivity using selective ion monitoring (SIM) techniques. During this process, the MS analysis time is focused only on analytes of specific masses, and all others are excluded. Even greater specificity may be obtained by analyte fragmentation, and monitoring both parent and one (SRM) or more product ions simultaneously (MRM), as reported in Figure 3.

This process is not the only approach suitable for analyte quantification, but it is certainly one of the most sensitive and selective. The essential ability of MRM is to detect a specific precursor ion, isolate it for collision-induced fragmentation, and detect a specific product ion following fragmentation.[43,44] All QqQ instruments may perform MRM analysis, but selection of the optimal platform will be dependent on the nature and quantity of analytes under investigation and will be especially related to the coupled separation system (conventional HPLC or UHPLC). A typical output relevant to these cases is shown in Figure 4.

Great improvement in selectivity and sensitivity of HPLC analyses is especially due to the introduction of nano-ESI, a highly efficient electrospray interface that permits use of flow rates at nanoliters level (<100 nL/min), characteristic of nano-HPLC separation systems.

There have been further ESI developments involving the use of lower mobile phase flow. Chowdury and Chait in 1991[45] showed that a

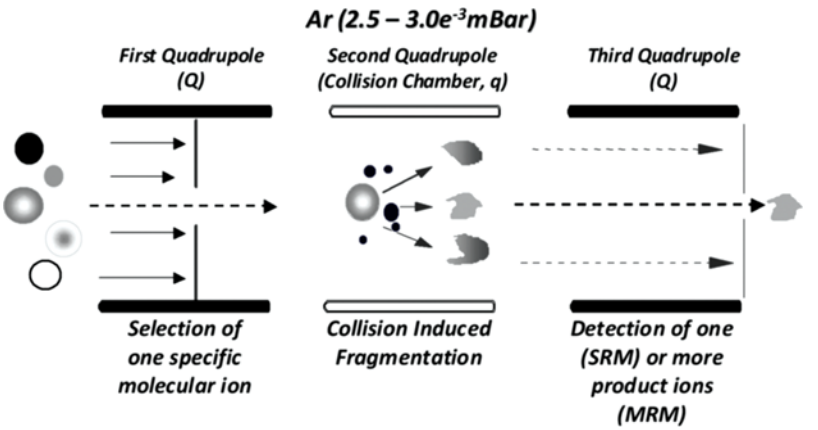

FIGURE 3 Triple quadrupole mass analyzer configuration operating in SRM mode (only one fragment) or in MRM mode (two or more fragments) (color figure available online.).

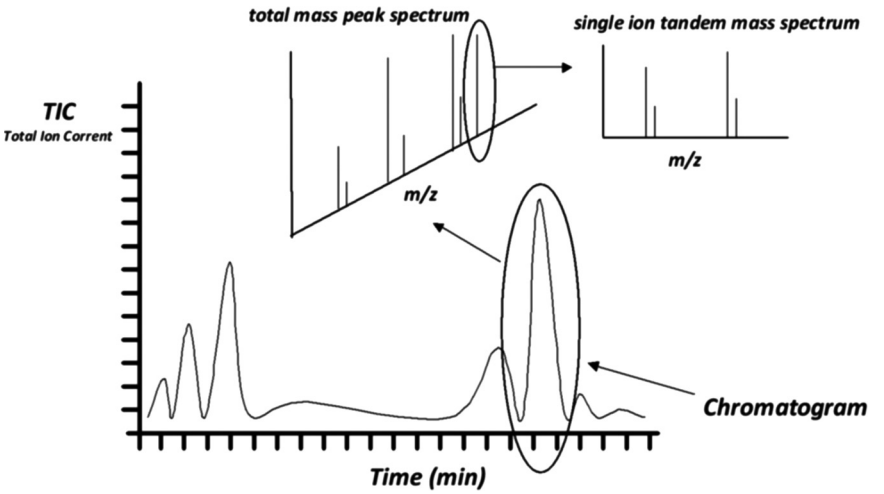

FIGURE 4 HPLC-MS data with total ion current chromatogram, total mass peak spectrum, and single ion tandem mass spectrum.

needle with a fine taper would allow aqueous solutions to be analyzed by ESI. The taper caused a higher electric potential at the capillary tip, facilitating droplet formation and consequently obtaining a more efficient ionization process, thus improving sensitivity. Wilm and Mann[46] used flow-rates lower than nanoliters (nL) per minute in their "nanospray" technique, employing capillaries with a tapered outlet of 1–2 μm inner diameter. A requirement for micro/nano-spray is a low inner diameter capillary outlet, generally incorporating a tapered tip and electrical contact via metal coating of the capillary tip, applied by gold deposition.

HPLC-MS/MS and nano-HPLC-MS/MS proved to be extremely sensitive and specific techniques for the analysis of pharmaceuticals (drugs, metabolites, and impurities). They play an important role in studying drug metabolism, discovering new drug candidates, and completely analyzing, identifying, and characterizing impurities and degradation products. In the future, technical advances in MS are expected to go on, especially to improve sensitivity and selectivity. The trend is toward further development of hybrid instruments such as quadrupole reflectron time of flight (Q-Re-TOF) that can be applied for quantitative analyses. Recently, the introduction of a novel mass analyzer, Orbitrap, became prominent as a development in instrument configuration, and in particular the mass spectrometer became less complex to use and more available for routine analyses.

The likely importance of proteomics in pharmaceutical development will have implications for MS as a detection technique, leading to further demand for high-resolution sequencing. Among these applications,

tandem mass spectrometry (MS/MS) is particularly suitable for drugs and metabolic studies, while high-resolution mass spectrometry (with FT-ICR or Re-TOF analyzer) is particularly suitable for biomarker discovery and validation.

Nuclear Magnetic Resonance (NMR)

Over the last two decades, considerable attention has been given to the development of hyphenated techniques, which combine improved separation technologies with nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). HPLC is now a well-developed and widely used technique for the separation of complex mixtures. NMR techniques, as well as MS, provide univocal structural information for the isolated compounds.

For the direct NMR interface, the "classic" probe was transformed into a flow-through probe by the introduction of a thin-walled Teflon capillary within a standard NMR tube, and spectra were recorded with sample rotation. Bayer et al.[47] employed a different probe specifically designed for the analysis of mixtures of known compounds in both on-flow and stopped-flow operation modes.

Comparison between spectra acquired using HPLC-NMR and conventional methods revealed that resolution in the latter case was better. The probes currently used are derived from that particular prototype. The first real sample analyzed by HPLC–NMR was military jet fuel with normal-phase columns and deuterated chloroform and freon 113.[48,49] In general, the use of normal-phase columns considerably restricted the field of application, but the use of reversed-phase columns caused additional problems in HPLC–NMR, because it is necessary to use mixtures of water/organic solvent modifier. A list of hyphenation methods was published in the 1980s, aiming to overcome the drawbacks of the HPLC–NMR coupling.[50–53] HPLC–NMR technology must deal with the interfacing, the flow-through probe design, and several analytical factors that can influence the instrumental response, such as sensitivity, solvent suppression, NMR- and HPLC-compatible solvents, and the chromatographic peak volume versus the NMR flow-cell volume [54].

When coupling HPLC with NMR, it is possible to operate in "on-flow mode": the sample is flowed continuously trough the NMR flow-cell during data acquisition, and the NMR spectrometer acts as a UV or MS detector in a chromatographic system, since the sample is measured without stopping the flow.

The result is typically displayed as a two-dimensional (2D) time-frequency plot with one-dimensional spectra for frequency field and retention time, similar to an HPLC–DAD chromatogram plot.[55] The

optimum flow-rate for continuous-flow NMR is usually chosen as a compromise between the rate for the best chromatographic resolution and the best NMR sensitivity.[56] The main problem is that ^{1}H NMR data acquisition and signal-to-noise for compounds at lower concentration level are unsatisfactory, and the direct acquisition of ^{13}C NMR spectra even for the main constituents is not possible.

For these reasons there are two other methods by which NMR measurements can be carried out under non-flowing or static conditions:

1. Using a valve to stop the elution when the analyte reaches the flow-cell within the radio frequency coil (stopped-flow mode)
2. Using sample loops for temporary storage of the individual analyte fractions from the chromatographic separation

After NMR data acquisition, the chromatographic run is restarted, and the procedure is repeated for the next analyte[57] (Figure 5).

A large number of chromatographic peaks (and consequently a large number of unknown sample constituents) can be studied by a separation procedure in several stopped-flow mode steps. In this acquisition mode, the frequent stops may influence the separation quality, and highly concentrated compounds may remain in the NMR detection cell (carryover effect). To bypass this problem, a novel loop storage mode was

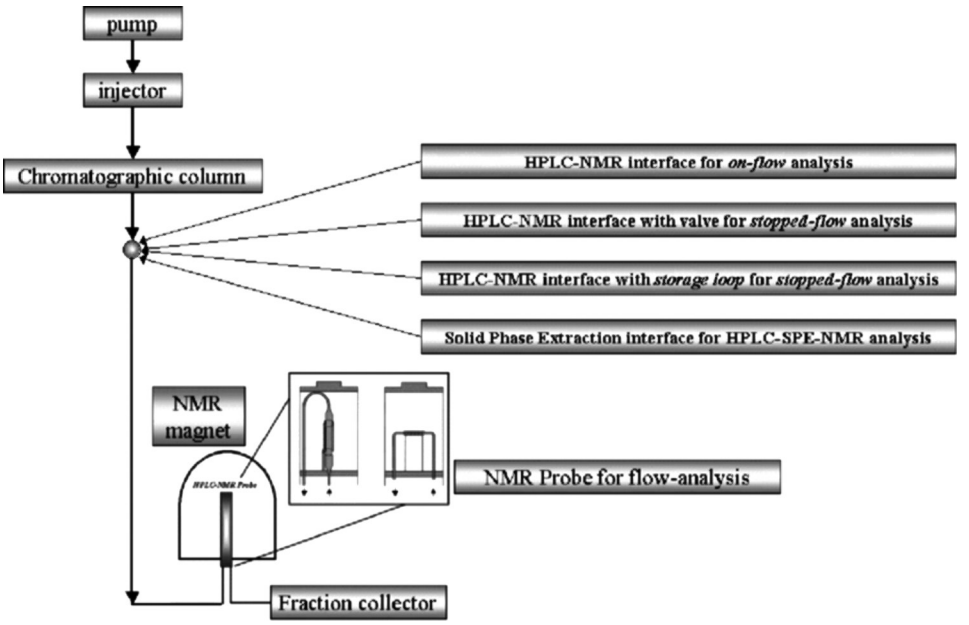

FIGURE 5 Various available HPLC–NMR configuration.

developed. The chromatographic run is not interrupted, but each analyte peak is stored in an individual capillary loop for NMR data acquisition in a subsequent stage. After each analyte measurement, the storage loop and the NMR flow cell can be washed automatically with a suitable solvent. To improve HPLC-NMR detection sensitivity, it is also possible to concentrate the samples, in order to achieve the highest analyte concentration in a minimum volume. In this way, solid-phase extraction (SPE) is a powerful technique for reproducible, rapid, selective, and sensitive sample preparation.[58]

The first automated on-line HPLC–SPE–NMR measurements were carried out on an extract of Greek oregano.[59] The separated peaks were post-column diluted with water, and trapped automatically on SPE cartridges. A drying step with nitrogen was used to remove all solvents that were used in the chromatographic separation. The analytes were then transferred with the deuterated solvent to the NMR flow-cell probe, and spectra were acquired. On-line LC–SPE–NMR for "peak parking" mode allows the use of normal protonated solvents for the HPLC run, and thus the need of solvent suppression is strongly reduced, or solvent suppression is even no longer necessary. This instrument configuration (HPLC–SPE–NMR) would provide an increase in terms of sensitivity and selectivity when compared to conventional HPLC–NMR. Recently, also the NMR instrumentations were improved by cooling the NMR RF coil and preamplifier electronics to cryogenic temperatures, while maintaining the sample at ambient temperature. This virtually eliminates the thermal electronic noise, which is generally associated with the initial steps of signal detection, and the coil quality-factor results to be increased.[60] An improvement in the signal-to-noise ratio by a factor of three to four was achieved.[61,62] The introduction of miniaturized solenoid micro-coils[63] is today one of the most promising developments in NMR probe design, besides cryogenic probes.

Infrared Spectroscopy (IR)

Coupling HPLC to IR systems can be done both off-line (after solvent removal) and on-line (using flow-through cells). The first case involves the use of interfaces that permit an improvement in sensitivity. However, the on-line approach provides major advantages, such as chromatographic resolution, real-time measurement, instrumental simplicity, low cost, possibility of using nonvolatile buffers,[64] and high-throughput analyses. On-line coupling allows measurement of IR analyte spectra without any orientation, crystallization, or oxidative (or chemical) degradation, which can occur when solvents are removed. Until now on-line IR has been limited by technical drawbacks that have restricted its applicability. The

most cited disadvantages of on-line HPLC-IR/FT-IR concern especially the two following:

1. Difficulties in correcting the background solvents absorption and presence of additives used for the mobile phases preparation
2. Low detection sensitivity due to reduced cell optical path-length to avoid signal saturation

Both problems are closely related, and it is easy to understand the corresponding limited number of research articles concerning this instrument configuration. Generally, to improve LODs and consequently LOQs, a series of technical innovations have been proposed, such as improved sensitivity detectors[65] and use of quantum cascade lasers (QCLs) as radiation sources.[66,67] See Table 2.

An important drawback of HPLC-IR is its low sensitivity. To improve the LOD, a capillary HPLC system was successfully interfaced with an FT-IR spectrometer,[73] using a micro-transmission flow-cell[74] with CaF_2 windows and low internal volume (7.5 nL) placed on a dedicated beam condenser. Recently QCLs have been employed as light sources in HPLC-IR measurements, having a great potential for the acquisition of group-specific chromatograms, and especially an important sensitivity improvement. QCL is still expensive and consequently rarely used. Dedicated flow-cells with low volumes have been described,[75] and have

TABLE 2 Applications of Isocratic On-Line Liquid chromatography–Fourier Transform Infrared (LC-FTIR)[68]

Technique	Analytical Column	Application	Reference
FT-IR spectrometer, ATR diamond flow cell (9 reflections)	300 × 7.8, 8 µm sulfonated styrene-divinylbenzene	Glucose and fructose in wine	[66]
FT-IR spectrometer, ATR diamond flow cell (9 reflections)	300 × 7.8, 8 µm sulfonated styrene-divinylbenzene	Carbohydrates, alcohols, and organic acids in wine	[69]
Microscope and FTIR spectrometer, germanium micro-ATR (1 reflection)	50 × 1.0, 3 µm ODS stationary phase	Succinylcholine chloride and methyl 3-hydroxybenzoate	[70]
FTIR spectrometer, Flow cell (CaF_2)	250 × 3.0, 5 µm ODS stationary phase	Peroxide-based explosives in solid samples	[71]
FTIR spectrometer, flow cell (BaF_2 and ZnSe)	250 × 2.0, 5 µm ODS stationary phase	Glycolic acid in cosmetics	[72]

been demonstrated to be useful to improve chromatographic resolution in HPLC-IR hyphenation.

ROLE OF CHEMOMETRIC DATA PROCESSING IN IMPROVING SENSITIVITY AND SELECTIVITY

For every analytical problem, a deep knowledge from the chemical standpoint is absolutely necessary, but sometimes it is not sufficient. In fact, even after optimizing sample pretreatment and instrumental performance (particularly in terms of sensitivity and selectivity), problems still remain in quantitative data processing when high complexity is faced. This is the case of multi-analyte analyses and the low number of standard samples available with respect to numerous variables influencing the analytical signals used for quantitative calibration. Moreover, even experimental choices in preparing standard samples may not be at all obvious, in that it is not so easy to make standard samples really representative of unknown samples when complex methodologies are faced. The need for statistical methods capable of obtaining the maximum information available from data is urgent.[76] Statistics help us to solve problems by simply working on numbers, once the chemical issues have been faced.

The type of statistics involved in complex analytical problems is multivariate statistics. The main chemometric tools particularly useful for separation and detection sciences are as follows:

- Experimental design
- Data exploration and pretreatment
- Data modeling for qualitative purposes, that is, classification
- Data modeling for quantitative purposes, that is, calibration through regression

Design of Experiments (DoE)

When complexity characterizes both instrumentation and unknown samples, it is difficult to make standard samples, forming the so-called training set, to be representative and capable of predicting reference known samples, forming the so-called test set, with high analytical performance. Chemometrics helps in this process, since it applies statistical methods to systematically vary input variables within predefined ranges, so that their effects on the output variables can be estimated and checked for significance. Several authors have applied DoE to liquid chromatography with mass-spectrometry detection,[77] electrochemical detection,[78] UV detection,[79] IR detection,[80] and fluorescence detection,[81] and to multi-analyte determinations in liquid chromatography.[82]

Exploration of Data: Principal Components Analysis (PCA)

If we start from a well-known and reliable training set and opportunely choose two principal components (PCs), containing at least 80% of explained variance and representative of those original variables that are significant in the chemical problem in object, we can easily visualize the objects forming the training set in score plots, while the role of original variables relevant to PCs is visualized in loading plots, as reported by Todeschini.[83] This kind of data exploration is called principal components analysis (PCA).

PCA has been extensively applied to data from LC analysis: HPLC-MS/MS and HPLC-ESI-HRMS/MS,[84] HPLC-NMR,[85] HPLC with spectrophotometric detection,[86] and HPLC-IR.[87]

Classification

Following a PCA, it is possible to model data by finding an equation relating analytical signals (responses) to original variables. When the response belongs to a particular class of samples, the resulting chemometric task is classification. Many examples of classification based on PC calculation and referring to HPLC data can be found in the literature, for instance HPLC-ICP-MS,[88], HPLC-NMR,[85] proteomics by HPLC,[89] peptide mapping by HPLC-ESI-MS,[90] HPLC-MS metabolic investigation,[91] and metabolic profiling.[92]

The various classification methods differ from each other in the applied algorithms. As for the multivariate classification methods, which are particularly suitable for separation-based analytical methodologies, two cases are particularly used:

1. Discriminant analysis (DA), based on an algorithm that compares *a* priori probabilities with a posteriori probabilities. Probabilities are calculated assuming that experimental data are normally distributed.
2. Soft independent models of class analogy (SIMCA), based on calculation of the distance between objects and as many PCA models as the number of classes of objects constituting the training set. SIMCA classification provides very easy-to-read outputs, called Coomans' plots. A generic Coomans' plot is shown in Figure 6.

The four sectors into which the plot is divided correspond to the four possible situations:

1. Objects are assigned neither to model A nor to model B (sector 1).
2. Objects are assigned to model B (sector 2).

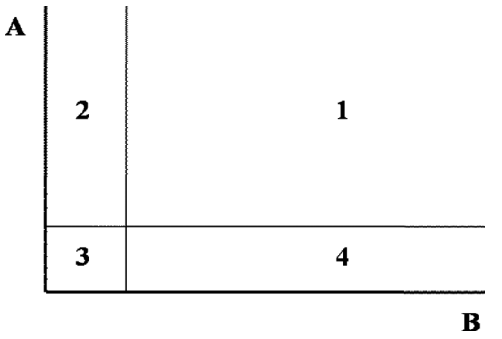

FIGURE 6 Coomans' plot relevant to two generic classes. A is the distance of objects from the model relevant to class A. B is the distance of objects from the model relevant to class A.

3. Objects are assigned both to model A and to model B (sector 3).
4. Objects are assigned to model A (sector 4).

Many applications of DA[77,89,93,94] and SIMCA[93,94] can be found in the literature relevant to HPLC. SIMCA is particularly useful when samples may belong to more than one class, or there is the possibility that objects do not belong to any class.

Calibration

Calibration is the basis of any quantitative analysis: a model is created by regression, starting from data collected for a training set; after that, prediction through the model allows calculation of unknown concentrations. Multivariate calibration relevant to HPLC data requires models based on PC calculation. Two main tools are used: principal components regression (PCR)[94] and partial least squares regression (PLS).[86,91] PLS is necessary when at least one of the following constraints applies:

1. The number of objects forming the training set is much lower than the number of measured variables.
2. The variables are strongly correlated with each other. This is typically the case of spectral variables.
3. Two or more responses are measured.

In all other cases, PCR may be applied. The chemometric approach also improves the selectivity and sensitivity in the most critical cases, such as for instance HPLC-IR hyphenation[95].

From the point of view of analytical performance, PCR and PLS enhance the quality obtained by optimizing sample treatment and instrumentation, but an open issue is still to be faced. As of now, no

validated formula is available to evaluate the absolute error relevant to predicted concentration.[96] Commercial software provides an estimate of error relevant to predicted values, but the reliability of this estimation still needs to be evaluated.

APPLICATIONS

Locatelli et al.[97] applied flow analyses for determining the molar absorptivity of two natural products from HPLC peak-area data. In this field, the analysis of secondary metabolites in plants is a difficult task, especially due to their chemical variety and diversity; low abundance and variability still within the same species; and the fact that sometimes they are also linked to different periods or seasons. It is estimated that 100.000–200.000 metabolites generally occur in the plant kingdom,[98] and considering the fact that many traditional herbal preparations contain not only one but also several medicinal plants, only highly selective and sensitive methods will be suitable for quality control analyses, as well as complete identification and quantification of unknown compounds and their metabolites.

Even if sensitivity is the disadvantage in using proton nuclear magnetic resonance analysis (^{1}H NMR) for this purpose, it is the only technique that deals with signals directly correlating with the amount of analytes in the sample.[99].

Recent hybrid technology developments that combine MS with FT-ICR with advanced ion trap technologies allow us to improve analytical performance. In particular, the exact molecular formula may be determined with high precision, trueness, resolution, and sensitivity.[100] Resolution values up to 700.000 and sensitivity at the attomole level may be achieved. The power of the high-resolution mass spectrometer is that it provides detailed structural information of MS/MS fragmentation, and at the same time it rapidly eliminates various possibilities from the list and pinpoints the sample's structure. Consequently, the major advantage derives from the coupling of highly hyphenated instrument configuration, such as HPLC-SPE-NMR-MS-FT-IR, permitting the complete, univocal, and accurate identification, quantification, and characterization of all constituents in the sample.

The power of combining very sensitive and selective separation technologies (especially with on-line extraction procedure) with spectroscopic techniques has been demonstrated for both quantitative and qualitative analyses of unknown compounds in complex matrices, not only in biological (and phytochemical) fields, but also in environmental and pharmaceutical applications.

CONCLUSIONS

The main potentiality of HPLC relates to its flexibility and its possibility of coupling with a very powerful detection method, that is MS (and especially tandem mass spectrometry, MS/MS), and with other novel detector devices such as NMR and IR, in order to obtain complete identification and quantification of the analytes, their metabolites, and structural characterization of unknown degradation products.

Another way to improve sensitivity and selectivity regards the use of on-line enrichment techniques. Coupling high-throughput sample preparation techniques with multiplexed HPLC-MS/MS leads to even faster analyses and the potential of interfacing HPLC-NMR with MS to give a HPLC-NMR-MS system, which allows the unequivocal identification of unknown compounds.

The use of micro-fluidic systems offers perspectives for miniaturized chip separations, and even the possibility of miniaturized mass spectrometers in the rather more distant future.

Multiple hyphenation techniques, such as HPLC-SPE-NMR-MS-FT-IR, represent the potential direction of a complete and robust method for the rapid de-replication of several real matrices analyses. In order to improve sensitivity and selectivity on different products discovery, deep extensions in hyphenation techniques are certainly required.

Finally, chemometrics may in general enhance analytical performance, and in particular selectivity and sensitivity, via statistical interpretation of experimental data. Multivariate tools for data processing extensively used in HPLC-based analytical methodologies are principal components analysis (PCA) for data exploration, discriminant analysis (DA) and soft independent models of class analogy (SIMCA) for data classification, and partial least squares regression (PLSR) for calibration aim to quantitative determinations.

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